Published in final edited form as:

Nat Struct Mol Biol. 2010 February ; 17(2): 165–172. doi:10.1038/nsmb.1765.

Structural basis for dsRNA recognition and interferon antagonism by Ebola VP35

Daisy W. Leung¹, Kathleen C. Prins², Dominika M. Borek³, Mina Farahbakhsh^{1,4}, JoAnn M. Tufariello², Parameshwaran Ramanan^{1,5}, Jay C. Nix⁶, Luke Helgeson^{1,4}, Zbyszek Otwinowski³, Richard B. Honzatko¹, Christopher F. Basler², and Gaya K. Amarasinghe¹

¹Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011, USA

²Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029, USA

³Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-8816, USA

⁴Biochemistry Undergraduate Program, Iowa State University, Ames, IA 50011, USA

⁵Biochemistry Graduate Program, Iowa State University, Ames, IA 50011, USA

⁶Advanced Light Source, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

Summary

The VP35 protein encoded by the highly pathogenic Ebola virus facilitates immune evasion by antagonizing antiviral signaling pathways, including those initiated by RIG-I like receptors. Here we report the crystal structure of Ebola VP35 interferon inhibitory domain (IID) bound to short double-stranded RNA (dsRNA), which reveals how VP35-dsRNA interactions contribute to immune evasion, and corresponding *in vivo* studies. Conserved basic residues in VP35 IID recognize the dsRNA backbone, whereas the dsRNA blunt ends are “end-capped” by a pocket of hydrophobic residues that mimics blunt end dsRNA recognition by RIG-I-like receptors. Residues that are critical for RNA binding are also important for interferon inhibition *in vivo*, but not for viral polymerase co-factor function of VP35. These results suggest that simultaneous recognition of dsRNA backbone and blunt ends provides a mechanism by which Ebola VP35 antagonizes host dsRNA sensors and immune responses.

Introduction

Ebola virus (EBOV) infections often result in death, reflecting an inability of the host immune response to control virus replication. This may arise, in part, from the ability of EBOV to suppress dendritic cell function and to promote macrophage cytokine production that forms the first line of immunological defense^{1–3}. Fatal cases also exhibit defective humoral responses to viral infection⁴. These observations are largely due to the subversion of the host innate

Corresponding author: Gaya K. Amarasinghe, Tel: (515) 294-3216, Fax: (515) 294-0453, amarasin@iastate.edu.

Accession codes: Coordinates and structure factors have been deposited in the Protein Data Bank under accession numbers 3L25 (native VP35 IID- 8 bp dsRNA complex), 3L26 (SeMet VP35 IID – 8 bp dsRNA), 3L27 (VP35 IID R312A mutant), and 3L28 (VP35 IID K339A mutant).

Author contributions: D.W.L., C.F.B. and G.K.A. designed research; D.W.L., K.C.P., D.M.B., M.F., J.M.T., P.R., J.C.N., L.A.H., Z.O., R.B.H. C.F.B., and G.K.A. performed research and analyzed data; D.W.L., C.F.B. and G.K.A. wrote the manuscript.

Methods: Methods and associated references are available in the online version of the paper.

immune system by EBOV, which also contributes substantially to high mortality rates associated with EBOV outbreaks⁵. RIG-I-like receptors (RLRs) are a central component of host innate immunity, which detect viral nucleic acids during infection and replication⁶⁻⁸. Preactivation of RIG-I alone can inhibit virus replication reducing virus growth up to ~1000-fold, suggesting that inhibition of RLRs plays a substantial role in EBOV propagation⁹. Recently it has been shown that RLRs recognize and are activated by a variety of RNA ligands, including 5'ppp and blunt ends of double stranded RNA (dsRNA)¹⁰⁻¹⁴. Viruses have also developed countermeasures to RLR-mediated antiviral pathways and the Ebola viral protein 35 (VP35) has recently emerged as a key viral component that can antagonize antiviral pathways activated by RLRs¹⁵⁻²³.

Multifunctional VP35 is also critical for viral replication^{17,20}, RNA silencing suppression²⁴, RNA-dependent protein kinase (PKR) inhibition²⁵, and nucleocapsid formation²⁶⁻²⁹. VP35 is composed of an N-terminal coiled-coil domain³⁰ and a C-terminal interferon inhibitory domain (IID)^{15,17,22} (reviewed in Basler and Amarasinghe³¹). The coiled-coil domain is required for several VP35-mediated functions, including viral replication and nucleocapsid formation³⁰. We and others have shown that VP35 binds dsRNA through its C-terminal IID, which is sufficient for interferon (IFN) inhibition^{15,17,22}. Our recent crystal structure of the VP35 IID revealed a cluster of conserved basic residues that are important for dsRNA binding³². Mutation of Arg312 in this cluster prevents dsRNA binding^{17,20,32} and VP35 proteins containing Arg312 mutations are defective in IFN inhibition, supporting a role of Arg312 in dsRNA-dependent IFN-inhibition. Consistent with these observations, infection by wildtype or VP35 R312A EBOV leads to differential expression of antiviral genes, including upregulation of IFN- α/β , RIG-I/MDA5, and STAT1 in cells infected with the mutant virus^{21, 23}. Moreover, growth rates of a mouse-adapted EBOV containing VP35 R312A mutation were greatly attenuated compared to wildtype EBOV¹⁹. Transfection of the VP35 R312A mutant into 293T cells overexpressing RIG-I results in reduced IFN inhibition upon subsequent challenge with Sendai virus^{17,20}. These studies underscore the importance of basic residues centered on Arg312 in VP35 IID. However, the mechanistic basis of how VP35 facilitates EBOV immune evasion, including the functional importance of the central basic patch as well as the role of dsRNA-independent and dsRNA-dependent VP35 mediated inhibition of the RLR pathway, is not well understood.

Here we present the crystal structure of *Zaire ebolavirus* VP35 IID bound to an 8 base pair (bp) dsRNA, together with the structures of two mutant proteins that cannot bind dsRNA; in these studies, we define the IFN inhibitory properties of VP35 on the basis of structural, biochemical, and cell biological studies. The structure of VP35 IID/dsRNA complex identified several conserved basic residues from VP35 IID that are important for protein-protein and protein-RNA interactions. Hydrophobic residues at the intersubdomain interface of VP35 IID interact with the blunt ends of dsRNA, forming a previously unrecognized "end-cap" that mimics RLR interactions with dsRNA. Analysis of mutant crystal structures of VP35 IID (R312A and K339A) reveals that changes in surface electrostatics at select locations in VP35 IID result in diminished dsRNA binding. Corresponding *in vivo* IFN- β promoter assays show a strong correlation among residues that are important for dsRNA binding and those required for full IFN inhibition. Interestingly, residues that are important for IFN inhibition are not essential for viral polymerase co-factor function of EBOV VP35, suggesting that these two functions are likely carried out by distinct regions within VP35 IID. Structurally pre-defined *in vitro* transcribed (IVT) RNAs (short IVT-dsRNA and IVT-hairpin RNAs) with base-paired stems that are similar in length to those used in our structural studies can activate RIG-I-dependent IFN- β production and IVT-dsRNA as short as 8 bp can activate IFN- β induction. Additionally, VP35 IID can compete with RIG-I C-terminal RNA binding domain for short IVT-dsRNA *in vitro* and overexpression of EBOV VP35 can inhibit IFN- β signal activated by IVT-dsRNA *in vivo*. Collectively, these studies support a mechanistic model, where dsRNA

recognition and sequestration by Ebola VP35 prevents activation of host RLRs. Our studies also suggest that VP35 is indispensable for efficient Ebola viral propagation due to its ability to antagonize multiple components in the IFN signaling pathways initiated by host RLRs.

Results

Central basic patch forms intermolecular interactions

The structure of EBOV VP35 IID in complex with an 8 bp dsRNA was determined using selenomethionine (SeMet) labeled and native proteins. The SeMet and native complexes crystallized in the space groups $P2_12_12_1$ and $P4_32_12$, respectively. Both structures were solved by molecular replacement to 2.0 Å and 2.4 Å resolution for SeMet and native complexes, respectively, using the ligand free structure as a search model (Fig. 1, Table 1). The corresponding unbiased map was used to build the dsRNA structure and the electron density is shown in Supplementary Fig. 1. Comparison of the two complex structures indicates that they are nearly identical with r.m.s.d. values < 0.5 Å for all backbone atoms; therefore, the highest resolution structure (SeMet) was used for all subsequent analyses.

Four VP35 IID molecules and one 8 bp dsRNA are observed in the crystallographic asymmetric unit. The complex structure exhibits two-fold non-crystallographic symmetry, where VP35 IID molecules A and B are equivalent to molecules C and D, respectively (Fig. 1a). The VP35 IID molecules in the complex show limited structural perturbations relative to the ligand-free structure (PDB: 3FKE; r.m.s.d. values < 0.5 Å). Molecules A and B (Fig. 1a-b) interact through a series of hydrogen bonds (Fig. 2a-b). Arg312 from molecule A forms hydrogen bonds with Gly270 O, Asp271 Oδ1, and Glu269 O of molecule B. Arg322 of molecule A forms hydrogen bonds with residues Glu262 Oε1 and Oε2, Glu269 Oε1 and Oε2 of molecule B. By symmetry, molecules C and D display similar interactions. Therefore, these conserved basic residues of molecules A and C form few solvent mediated contacts with dsRNA. Other residues from molecules A and C (Pro233, Thr237, Phe239, Ser272, Gln274, Cys275, Ile278, Ala306, Lys309, Ser310, and Ile340) form van der Waals and water mediated contacts with dsRNA. In contrast, the sidechains of Arg312 and Arg322 in molecules B and D form direct contacts with the phosphodiester backbone of dsRNA (Fig. 2c-d) (Arg312 NH1/NH2 to U5 O1P/O2P and G8 O2P; and Arg322 NH2 to G6 O1P and C7 O2P). In the absence of base specific contacts, it is likely that the interactions observed between VP35 IID central basic patch and the 8 bp dsRNA are independent of the nucleotide sequence. Collectively, the protein-protein and protein-RNA interactions observed in the complex suggest that the central basic patch may function in both dsRNA-independent and dsRNA-dependent VP35-mediated functions. Interestingly, the conformations of the side and main chains are similar, which suggests that the functional versatility of the residues in EBOV VP35 IID is mediated through changes in sidechain conformations (Supplementary Table 1).

Isothermal titration calorimetry (ITC) experiments reveal that the 8 bp dsRNA used in the structural studies binds VP35 IID with high affinity ($K_D = 500$ nM) and a stoichiometry of 4:1 VP35 IID to dsRNA, which is consistent with the number of molecules in the crystallographic complex (Fig. 1c). ITC assays with IVT-8 bp dsRNA, containing 5'ppp revealed that VP35 IID binds with higher affinity ($K_{D,1} = 30$ nM, $K_{D,2} = 2.2$ μM with 1 and 3 binding sites, respectively), which retains the stoichiometry observed in the complex structure (Supplementary Fig. 2a). However, VP35 IID does not bind single stranded RNA (Fig. 1d).

We previously identified two distinct conserved basic patches located in the α-helical and β-sheet subdomains of VP35 IID, termed first and central basic patch, respectively³². Gel-shift analyses indicate that only the latter patch is important for dsRNA interactions. Consistent with this finding, only residues from the central basic patch interact with dsRNA in the VP35 IID/dsRNA complex structure. The importance of basic residues in dsRNA recognition was further

assessed by ITC studies. Mutation of Arg305 or Lys319 to alanine results in a reduction in the binding affinity relative to wildtype protein by a factor of 3- to 5-fold (Supplementary Fig. 2b). In contrast, proteins containing an R312A, R322A, or K339A mutation failed to bind dsRNA (Supplementary Fig. 2c). These results are consistent with important intermolecular contacts between VP35 IID and the dsRNA observed in the complex structure, where Arg312, and Arg322 from molecule B (and D) participate in extensive interactions with dsRNA. Interestingly, Lys339 from molecule B does not make direct contacts with the dsRNA (see discussion below). Collectively, these results suggest that Arg305, Lys309, and Lys319 cause limited perturbations to binding, while Arg312, Arg322, and Lys339 are essential for dsRNA-dependent functions.

Central basic patch VP35 mutant structures

We previously reported that mutation of select residues within the central basic patch is unlikely to cause major structural changes based on NMR chemical shift mapping³². Therefore, it was not immediately evident why select single residue mutations (for example, R312A, R322A, or K339A) lead to a complete loss of dsRNA binding or why surrounding residues in the central basic patch cannot compensate for the mutation. In order to address these questions, we solved the crystal structures of R312A and K339A by molecular replacement to 2.00 Å and 2.40 Å, respectively (Table 1). Overall, both mutants maintain the backbone conformation of wildtype VP35 IID (PDB: 3FKE, molecule B was used for comparisons) and of VP35 IID in complex with dsRNA. Superposition of R312A or K339A with wildtype VP35 IID displays r.m.s.d. values of < 1.0 Å and < 0.5 Å change in the overall structure and in the α -helical/ β -sheet subdomain alignments, respectively (Supplementary Fig. 3a). Comparison of sidechain conformations, particularly near the central basic patch, revealed no direct correlation between loss of dsRNA binding and basic residue mutations (Supplementary Fig. 3b). For example, the R312A mutant protein structure is nearly identical in conformation to the wildtype structure. Electrostatic distribution of solvent accessible surfaces of mutant proteins revealed appreciable differences near regions that make contact with protein and dsRNA in the complex, suggesting that observed functional differences are due to change in surface charge distribution at specific sites (Fig. 3a-c). In contrast, evaluation of the *in silico* modeling of R305A, K309A, and K319A mutant structures suggest that these mutations are unlikely to cause substantial changes in the electrostatic surface within the interaction “footprint” defined by the VP35 IID/dsRNA complex (Supplementary Fig. 4). These results support a model where Arg312, Arg322, and Lys339 make critical contributions to dsRNA recognition and binding while Arg305, Lys309, and Lys319 enhance the key interactions (Fig. 3d). In the crystal structure of the complex, Arg312 or Arg322 makes direct RNA contacts, while Lys339 does not directly bind to dsRNA. Comparison of the K339A mutant structure to the wildtype VP35 IID structure revealed that the Lys339 sidechain coordinates the carboxylate group of the terminal residue, Ile340 (Supplementary Fig. 5), leading to charge neutralization. Mutation of Lys339 exposes the carboxy terminus, which is likely to introduce unfavorable interactions near the central basic patch and may lead to reduced dsRNA binding.

Hydrophobic residues “end-cap” short dsRNA

In the structure of the VP35 IID/dsRNA complex, several conserved hydrophobic residues at the intersubdomain interface form a previously unrecognized “end-cap” that interacts with the dsRNA ends (Fig. 4). Hydrogen bonds form between Gln274 N ϵ and C1 O4', and Ile340 OXT and C1 N4. Additional protein/dsRNA interactions include: electrostatic interactions between Lys282 N ζ and G8 O2P, and Arg322 N ϵ and C7 O2P.; van der Waals contacts between: Phe239 C ζ and C ϵ 2 to C6; Gln274 C γ to C6 O4'; Ile278 C δ 1 to G8 N1 and C6; Gln279 C δ to G8 O3'; Gln279 O ϵ 1 to G8 C2', C3', and O3'; Lys282 C δ to G8 O2P and O5'; and Lys288 C ϵ to G8 O2P. Recognition of short dsRNA by RLRs leads to potent activation of antiviral signaling pathways, including IFN- β production^{11,13}. Extensive interactions observed in our VP35/

dsRNA complex structure strongly suggest that VP35 IID is able to recognize blunt end conformations of short duplex RNA similar to those observed for RLRs^{14,33}.

We tested the importance of the “end-cap” interaction in dsRNA binding through mutation of residues that form the “end-cap” structure. ITC-based RNA binding assays for F235A and F239A revealed that mutation of Phe239 but not Phe235 results in a complete loss of dsRNA binding (Supplementary Fig. 6). These results are consistent with the structure of the complex, where Phe239 is part of the “end-cap” and forms van der Waals contacts with both dsRNA and terminal Ile340 from the β -sheet subdomain. Phe235 is outside the “end-cap” structure. Solution NMR analysis of the F239A mutant reveals that the mutation causes modest chemical shift perturbations (within 6–8 Å of the site of mutation), where a majority of the structure is largely unperturbed (data not shown). Together, these results suggest that limited structural changes are caused by mutation of “end-cap” residues. Therefore, the observed loss of dsRNA binding is likely due to the loss of intermolecular contacts between the sidechains of VP35 and the blunt ends of dsRNA.

Shape and charge complementarity in the complex

Shape and charge complementarity analyses support protein-protein and protein-RNA interactions observed in the complex structure. The calculated shape correlation statistics (Sc, a measure of the geometric match of two interacting surfaces)³⁴ are 0.61, 0.68, and 0.70 for dsRNA/molecule A, dsRNA/molecule B, and molecule A/molecule B, respectively. For reference, a Sc correlation of 1.0 indicates perfect complementarity, while most antibody-antigen complexes display Sc values of ~ 0.75 ³⁴. Similarly, examination of surface electrostatics also supports the intermolecular interactions observed in the structure. Approximately 1650 Å² are buried through interactions between VP35 IID proteins and the 8 bp dsRNA. Of the ~ 6300 Å² total available surface area for each VP35 IID molecule in the complex, about 400 Å² is contacting the dsRNA. In contrast, ~ 1400 Å² are in contact at the protein-protein interface, suggesting that protein-protein interactions make substantial contributions toward the complex formation. These protein-protein interactions are more important than those seen at the crystallographic dimer interface for wildtype VP35 IID³². Together, these data highlight the ability of VP35 IID to facilitate intermolecular interactions in a context dependent manner with limited backbone conformational changes. Given that the same VP35 IID-dsRNA complex appears in two different space groups, crystal packing has little or no effect on the interactions observed in the complex. This notion is further supported by solution NMR data of VP35 IID bound to dsRNA, where residues displaying the largest chemical shift differences upon RNA addition are involved in dsRNA recognition in the crystal structure (data not shown).

dsRNA-independent inhibition of IFN- β by VP35

In order to test dsRNA-independent IFN- β inhibition by VP35, we measured RIG-I CARD-mediated activation of IFN- β . RIG-I CARD domains when overexpressed activate the IFN- β promoter¹². This method of IFN- β promoter activation presumably does not require RNA ligands to activate antiviral signaling. Transfection of wildtype VP35 reduces IFN- β response generated by RIG-I CARD domains by about 65%. Mutation of residues involved in the dsRNA “end-cap” recognition (F239A and H240A) also suppress IFN- β promoter activation to levels similar to wildtype VP35 (Supplementary Fig. 7a). In contrast to wildtype and “end-cap” mutants, central basic patch mutants, R312A, R322A, and K339A, display reduced suppression of IFN- β promoter activation (Supplementary Fig. 7b). Interestingly, “end-cap” residues only display protein-dsRNA intermolecular interactions in the complex structure and they are not involved in protein-protein interactions such as those observed for the central basic patch residues (Figs. 2 and 4). These results demonstrate that when the IFN- β promoter is activated by a dsRNA-independent mechanism, VP35 is also able to partially suppress this activation.

This residual suppression does not require dsRNA binding by VP35 as F239A and H240A mutants cannot bind dsRNA. Therefore, the reduced IFN- β inhibition displayed by central basic patch residues, Arg312, Arg322, and Lys339, in this assay is likely due to protein-protein interactions mediated by VP35. However, the relative contributions to dsRNA-dependent and -independent functions cannot be directly determined as residues in the central basic patch are involved in both types of interactions.

dsRNA-dependent inhibition of IFN- β activation by VP35

Multifunctional Ebola VP35 is a cofactor for the viral polymerase L in addition to its role as an IFN antagonist. Therefore, we performed a series of *in vivo* experiments to evaluate the importance of residues that show intermolecular contacts in the complex structure. To this end, the viral polymerase cofactor function of VP35 IID was assessed in the context of full-length VP35 expressed in 293T cells. The viral transcription/replication complex is reconstituted by coexpression of EBOV NP, VP35, VP30 and L proteins, such that the complex transcribes and replicates a coexpressed model viral genome encoding only reporter genes³⁵. VP35 proteins containing mutations in the “end-cap” (F239A) or the central basic patch (R305A, K309A, R312A, K319A, R322A or K339A) were functional in a “minigenome” assay, which measures the activity of the viral RNA polymerase complex. Absolute levels of activity, however, showed some variation. These differences were either due to reduced expression level of some mutant VP35 proteins relative to wildtype VP35 (Table 2, Supplementary Fig. 8) or due to different levels of polymerase co-factor activity of the mutants. The latter possibility is currently under further characterization. However, our current data clearly demonstrate that mutation of residues important for dsRNA binding does not impair function in the replication/mini-genome assay. Interestingly, mutant F235A was nonfunctional, despite expression levels comparable to wildtype VP35. H240A also did not exhibit activity, but its poor level of expression may explain this absence of activity (data not shown). Together, these data suggests that the ability of VP35 to function as a viral polymerase cofactor is not dependant on the residues that are important for dsRNA binding.

We next characterized IFN-inhibition by EBOV VP35. To this end, we tested the inhibition of Sendai virus-mediated activation of the IFN- β promoter by wildtype and mutant EBOV VP35 proteins, which contained mutations within the IID (Fig. 5, Supplementary Fig. 9). These results revealed that “end-cap” and central basic patch residues are important in the inhibition of IFN activity, but not those from the first basic patch, as VP35 mutants that failed to bind dsRNA (F239A, R312A, R322A, and K339A) were also unable to suppress the IFN- β promoter relative to wildtype VP35 (Table 2, Supplementary Fig. 9). Additional residues tested, including F235A, R305A, K309A, and K319A, displayed reduced but measurable dsRNA binding activity and were able to suppress IFN- β reporter expression, but did so less efficiently than wildtype VP35. These results show a strong correlation with dsRNA binding data.

The data presented above support a model where dsRNA recognition by EBOV VP35 leads to dsRNA sequestration and may result in substantially diminished activation of cellular RLRs. In order to further test this model, the ability of VP35 to bind to RIG-I ligands using IVT-dsRNA that contains the 5'ppp motif was characterized^{11,13}. Corresponding ITC data, shown in Supplementary Fig. 2, revealed that VP35 IID can bind IVT-8 bp dsRNA with affinities similar to those observed for RIG-I/dsRNA binding¹¹. Interestingly, these binding affinities are higher than those observed for chemically synthesized 8 bp dsRNA that contain a 5' OH motif (Fig. 1C). Next, the ability of EBOV VP35 IID to bind and to compete for IVT-dsRNA in the presence of the RIG-I C-terminal domain was assessed. Using 1H/15N labeled VP35 IID, we monitored 2-D HSQC NMR signals for VP35 IID protein alone, as well as VP35 IID protein upon the addition of either dsRNA, dsRNA + RIG-I C-terminal domain (competition experiment), or RIG-I C-terminal domain (negative control). As shown in Fig. 6a, addition of

dsRNA reduces the NMR signal intensity of VP35 IID due to an increase in molecular size upon binding dsRNA (average 0.36 $\pm$ 0.1). Interestingly, addition of RIG-I C-terminal domain, which has been shown to bind dsRNAs similar in length and structure to those used in our studies^{33,36}, into the binary complex at equimolar concentrations does not lead to a complete reversal of peak intensities for VP35 IID (average intensity 0.42 $\pm$ 0.1). This data suggests that the RIG-I C-terminal domain cannot completely displace (and therefore, compete with) EBOV VP35 IID for dsRNA binding. VP35 IID peak intensities were also measured upon addition of RIG-I C-terminal domain as a negative control (average intensity 0.92 $\pm$ 0.1), which had no effect on the NMR signals of VP35 IID.

Structurally pre-defined dsRNAs were used to activate RIG-I specific signaling cascades in order to examine the effect on IFN- β promoter activation by VP35. Shown in Fig. 6b, IVT-dsRNA and IVT-hairpin RNAs (similar to those used by Schmidt *et al.*¹³), including stems as short as those containing 8 bp, can activate the IFN β promoter in 293T cells transfected with a RIG-I expression plasmid. Interestingly, the IVT-hairpin RNA consistently activates the IFN β promoter to a higher level than the IVT-8 bp dsRNA. Our results also show that a 5'-ppp was required for dsRNA-mediated activation IFN- β activation, which is consistent with previous reports (Fig. 6b). Co-transfection of wildtype EBOV VP35 inhibits RIG-I activation of the IFN- β promoter. Collectively, our data suggests that: 1) the IVT-dsRNA with lengths similar to that used in our structural study can activate IFN- β activity, 2) the IFN- β promoter activation is specific to RIG-I, and 3) VP35 can inhibit IVT-dsRNA-dependent activation by RIG-I.

Discussion

The combination of structural, biochemical, and cell biological studies described here provide a strong correlation between the ability of VP35 IID to bind dsRNA and to inhibit IFN- β promoter activation. Specifically, we show that residues in the central basic patch and those that form the “end-cap” in VP35 IID are important for dsRNA binding. Crystal structures of R312A and K339A show minor structural perturbations in the main chain, but reveal substantial changes in surface electrostatics near the dsRNA binding site, suggesting sidechain rearrangements may be responsible for reduced dsRNA binding. We show that *in vitro* transcribed RNA (IVT-8 bp dsRNA and IVT-hairpin) as short as 8 bp can activate the IFN- β promoter in a RIG-I specific manner, which is consistent with previous reports^{11,13}. We also show that IFN- β activation requires the presence of a 5'-ppp and that this activation is dependent on the RLR pathway.

Corresponding *in vivo* studies suggests that many residues that are important for dsRNA binding and for inhibition of IFN- β promoter may also function through additional protein-protein interactions, which may be important for downstream signaling. Specifically, we show that dsRNA-independent IFN- β activation is significantly diminished when central basic patch residue mutants are tested, but the “end-cap” mutants are functional in the same assay, providing further support for the existence of dsRNA-independent IFN antagonism by VP35.

RLRs¹⁸ and RNA-dependent protein kinase PKR²⁵ are important antiviral RNA sensors that can limit EBOV replication. Preactivation of RIG-I decreases EBOV replication, which supports the significance of RIG-I signaling toward EBOV pathogenesis⁹. Short dsRNAs and dsRNA containing 5'ppp are putative activators of RLRs and PKR³⁷⁻⁴¹. Comparison of our structure to the RLR LGP2 in complex with dsRNA reveals that both VP35 and LGP2 recognize similar structural features in dsRNA (Supplementary Fig. 10)^{33,40}. RIG-I, MDA5, and PKR also bind to and are activated by blunt end RNAs, suggesting that this recognition mode, observed in the LGP2/dsRNA complex, may be commonly employed by dsRNA-sensing pattern recognition receptors^{7,33,36,38,40,41}. VP35 IID can bind IVT-8 bp dsRNA with

affinities similar to those previously observed for RIG-I^{13,33,36}. Interestingly, the RNA binding domain at the C-terminus of RIG-I and the EBOV VP35 IID both exhibit higher affinities for 5'ppp dsRNA than 5'OH dsRNA of similar length and sequence, supporting the proposal that dsRNA containing 5'ppp are more potent activators of the RIG-I pathway. However, it is important to note that 5'OH dsRNA can also bind the C-terminal domain of RIG-I^{13,33,36} and VP35 (unpublished data DWL, GKA, CFB). Previously published data as well as our own observations support a model, where wide variation in the affinities for different RNA ligands, result in a greater response range that corresponds to the relative availability of these ligands. For example, RLRs bind with higher affinity for 5'ppp containing dsRNA (e.g. 10-100 nM for RIG-I, MDA-5, and LGP2)^{13,33,36}, but they also bind dsRNA lacking 5'ppp (e.g. 10-100 nM for RIG-I and ~70 nM for LGP2)^{13,33,36}. Our data reported here for VP35 and those previously characterized for RLRs, indicate that for a given ligand (either 5'ppp dsRNA or 5'OH dsRNA), the affinities of VP35 IID and RLR C-termini are similar. Therefore, given that large amounts of VP35 would be produced in EBOV-infected cells, VP35 likely competes with RIG-I for these RNA ligands. Moreover, full length VP35 is a trimer, which will likely display even higher binding affinities due to multivalency. It is interesting to note that the relative affinities for different RNA ligands qualitatively corresponds to their availability in infected cells, where more dsRNA is likely available than 5'ppp dsRNA ends. The ability of cellular RLRs to respond to a wide range of ligand affinities enable these key antiviral receptors to detect multiple viral signals, which would enable signal integration at RIG-I, leading to a more potent downstream signal and activation of IFNs. Therefore, competition between Ebola VP35 IID and cellular RNA sensors for similar RNA ligands emerges as a potential mechanism for facilitating VP35-mediated immune evasion. Mechanisms described in our study for dsRNA-dependent IFN antagonism by VP35 is likely to be an important contributor to Ebola viral pathogenesis as the "end-cap" mutants are unable to suppress dsRNA-dependent pathways yet can suppress dsRNA-independent activation as shown in the RIG-I CARD assays.

VP35 is part of a growing list of viral antagonists of host immunity that mimic cellular components. In addition to dsRNA-dependent activity described in this study, multifunctional VP35 utilizes dsRNA-independent functions that target other elements in the RLR signaling pathway. This may be reflected by the residual inhibition seen in our experiments using the RIG-I CARD domain to activate the IFN- β promoter, where activation of signaling by the RIG-I CARD domain is likely independent of dsRNA, and both wild-type and dsRNA binding mutant VP35s retain partial inhibitory functions. Similarly, both wildtype and R312A mutant VP35 proteins can partially inhibit IFN- α/β production induced by Sendai viral infection or by overexpression of IPS-1, TBK-1, or IKK ϵ ¹⁷. VP35 also associates with IRF-3 kinases and competes for the IRF-3 substrate binding site on IKK ϵ /TBK-1 in order to prevent IRF-3 phosphorylation⁴². Therefore, mechanisms that target additional elements within the RIG-I pathway likely contribute to EBOV immune evasion. Full knowledge of interactions between host factors and Ebola VP35 and future studies that characterize these interactions will facilitate the development of vaccines and antivirals against an indispensable component from a highly pathogenic virus and a potential agent of bioterrorism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank ISU Biotechnology Facilities and Drs. J. Hoy, N. Pohl, and D. B. Fulton for providing access to instrumentation and support. We also thank Drs. M. Nilsen-Hamilton and M. Shogren-Knaak for discussions; J. Binning and Dr. T. Wang for reading the manuscript; L. Tantral, D. Peterson, and C. Brown for lab assistance; and Drs. S. Ginell, N. Duke, F. Rotella, M. Cuff, and J. Lazarz at APS Sector 19. Use of Argonne National Laboratory Structural Biology Center beamlines at the Advanced Photon Source, was supported by the U.S. D.O.E. under contract

DE-AC02-06CH11357. This work is supported by NIH grants (1F32AI084324 to D.W.L., R01GM053163 to Z.O., R01NS010546 to R.B.H., R01AI059536 to C.F.B., and R01AI081914 to G.K.A.), MRCE Developmental Grant (U54AI057160-Virgin(PI) to G.K.A.), and Roy J. Carver Charitable Trust (09-3271 to G.K.A.).

References

1. Bosio CM, et al. Ebola and Marburg viruses replicate in monocyte-derived dendritic cells without inducing the production of cytokines and full maturation. *J Infect Dis* 2003;188:1630–8. [PubMed: 14639532]
2. Mahanty S, et al. Protection from lethal infection is determined by innate immune responses in a mouse model of Ebola virus infection. *Virology* 2003;312:415–24. [PubMed: 12919746]
3. Bray M, Geisbert TW. Ebola virus: the role of macrophages and dendritic cells in the pathogenesis of Ebola hemorrhagic fever. *Int J Biochem Cell Biol* 2005;37:1560–6. [PubMed: 15896665]
4. Baize S, et al. Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in Ebola virus-infected patients. *Nat Med* 1999;5:423–6. [PubMed: 10202932]
5. Feldmann H, Wahl-Jensen V, Jones SM, Stroher U. Ebola virus ecology: a continuing mystery. *Trends Microbiol* 2004;12:433–7. [PubMed: 15381189]
6. Matsukura S, et al. Role of RIG-I, MDA-5, and PKR on the expression of inflammatory chemokines induced by synthetic dsRNA in airway epithelial cells. *Int Arch Allergy Immunol* 2007;143:80–3. [PubMed: 17541283]
7. Hausmann S, Marq JB, Tapparel C, Kolakofsky D, Garcin D. RIG-I and dsRNA-induced IFN β activation. *PLoS ONE* 2008;3:e3965. [PubMed: 19115016]
8. Saito T, Gale M Jr. Principles of intracellular viral recognition. *Curr Opin Immunol* 2007;19:17–23. [PubMed: 17118636]
9. Spiropoulou CF, et al. RIG-I activation inhibits ebolavirus replication. *Virology* 2009;392:11–5. [PubMed: 19628240]
10. Schlee M, et al. Approaching the RNA ligand for RIG-I? *Immunol Rev* 2009;227:66–74. [PubMed: 19120476]
11. Schlee M, et al. Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* 2009;31:25–34. [PubMed: 19576794]
12. Yoneyama M, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 2004;5:730–7. [PubMed: 15208624]
13. Schmidt A, et al. 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proc Natl Acad Sci U S A* 2009;106:12067–72. [PubMed: 19574455]
14. Li X, et al. Structural basis of double-stranded RNA recognition by the RIG-I like receptor MDA5. *Arch Biochem Biophys* 2009;488:23–33. [PubMed: 19531363]
15. Basler CF, et al. The Ebola virus VP35 protein inhibits activation of interferon regulatory factor 3. *J Virol* 2003;77:7945–56. [PubMed: 12829834]
16. Basler CF, et al. The Ebola virus VP35 protein functions as a type I IFN antagonist. *Proc Natl Acad Sci U S A* 2000;97:12289–94. [PubMed: 11027311]
17. Cardenas WB, et al. Ebola virus VP35 protein binds double-stranded RNA and inhibits alpha/beta interferon production induced by RIG-I signaling. *J Virol* 2006;80:5168–78. [PubMed: 16698997]
18. Habjan M, et al. Processing of genome 5' termini as a strategy of negative-strand RNA viruses to avoid RIG-I-dependent interferon induction. *PLoS One* 2008;3:e2032. [PubMed: 18446221]
19. Hartman AL, et al. Inhibition of IRF-3 activation by VP35 is critical for the high level of virulence of ebola virus. *J Virol* 2008;82:2699–704. [PubMed: 18199658]
20. Hartman AL, Dover JE, Towner JS, Nichol ST. Reverse genetic generation of recombinant Zaire Ebola viruses containing disrupted IRF-3 inhibitory domains results in attenuated virus growth in vitro and higher levels of IRF-3 activation without inhibiting viral transcription or replication. *J Virol* 2006;80:6430–40. [PubMed: 16775331]
21. Hartman AL, Ling L, Nichol ST, Hibberd ML. Whole-genome expression profiling reveals that inhibition of host innate immune response pathways by Ebola virus can be reversed by a single amino acid change in the VP35 protein. *J Virol* 2008;82:5348–58. [PubMed: 18353943]

22. Hartman AL, Towner JS, Nichol ST. A C-terminal basic amino acid motif of Zaire ebolavirus VP35 is essential for type I interferon antagonism and displays high identity with the RNA-binding domain of another interferon antagonist, the NS1 protein of influenza A virus. *Virology* 2004;328:177–84. [PubMed: 15464838]
23. Kash JC, et al. Global suppression of the host antiviral response by Ebola- and Marburgviruses: increased antagonism of the type I interferon response is associated with enhanced virulence. *J Virol* 2006;80:3009–20. [PubMed: 16501110]
24. Haasnoot J, et al. The Ebola virus VP35 protein is a suppressor of RNA silencing. *PLoS Pathog* 2007;3:e86. [PubMed: 17590081]
25. Schumann M, Gantke T, Muhlberger E. Ebola virus VP35 antagonizes PKR activity through its C-terminal interferon inhibitory domain. *J Virol* 2009;83:8993–7. [PubMed: 19515768]
26. Huang Y, Xu L, Sun Y, Nabel GJ. The assembly of Ebola virus nucleocapsid requires virion-associated proteins 35 and 24 and posttranslational modification of nucleoprotein. *Mol Cell* 2002;10:307–16. [PubMed: 12191476]
27. Shi W, et al. A filovirus-unique region of Ebola virus nucleoprotein confers aberrant migration and mediates its incorporation into virions. *J Virol* 2008;82:6190–9. [PubMed: 18417588]
28. Johnson RF, McCarthy SE, Godlewski PJ, Harty RN. Ebola virus VP35-VP40 interaction is sufficient for packaging 3E-5E minigenome RNA into virus-like particles. *J Virol* 2006;80:5135–44. [PubMed: 16698994]
29. Noda T, et al. Ebola virus VP40 drives the formation of virus-like filamentous particles along with GP. *J Virol* 2002;76:4855–65. [PubMed: 11967302]
30. Reid SP, Cardenas WB, Basler CF. Homo-oligomerization facilitates the interferon-antagonist activity of the ebolavirus VP35 protein. *Virology* 2005;341:179–89. [PubMed: 16095644]
31. Basler CF, Amarasinghe GK. Evasion of interferon responses by Ebola and Marburg viruses. *J Interferon Cytokine Res* 2009;29:511–20. [PubMed: 19694547]
32. Leung DW, et al. Structure of the Ebola VP35 interferon inhibitory domain. *Proc Natl Acad Sci U S A* 2009;106:411–6. [PubMed: 19122151]
33. Li X, et al. The RIG-I-like Receptor LGP2 Recognizes the Termini of Double-stranded RNA. *J Biol Chem* 2009;284:13881–91. [PubMed: 19278996]
34. Lawrence MC, Colman PM. Shape complementarity at protein/protein interfaces. *J Mol Biol* 1993;234:946–50. [PubMed: 8263940]
35. Muhlberger E, Weik M, Volchkov VE, Klenk HD, Becker S. Comparison of the transcription and replication strategies of marburg virus and Ebola virus by using artificial replication systems. *J Virol* 1999;73:2333–42. [PubMed: 9971816]
36. Pippig DA, et al. The regulatory domain of the RIG-I family ATPase LGP2 senses double-stranded RNA. *Nucleic Acids Res* 2009;37:2014–25. [PubMed: 19208642]
37. Kato H, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006;441:101–5. [PubMed: 16625202]
38. Saito T, Gale M Jr. Differential recognition of double-stranded RNA by RIG-I-like receptors in antiviral immunity. *J Exp Med* 2008;205:1523–7. [PubMed: 18591413]
39. Nallagatla SR, et al. 5'-triphosphate-dependent activation of PKR by RNAs with short stem-loops. *Science* 2007;318:1455–8. [PubMed: 18048689]
40. Takahasi K, et al. Solution structures of cytosolic RNA sensor MDA5 and LGP2 C-terminal domains: identification of the RNA recognition loop in RIG-I-like receptors. *J Biol Chem* 2009;284:17465–74. [PubMed: 19380577]
41. Murali A, et al. Structure and function of LGP2, a DEX(D/H) helicase that regulates the innate immunity response. *J Biol Chem* 2008;283:15825–33. [PubMed: 18411269]
42. Prins KC, Cardenas WB, Basler CF. Ebola virus protein VP35 impairs the function of interferon regulatory factor-activating kinases IKKepsilon and TBK-1. *J Virol* 2009;83:3069–77. [PubMed: 19153231]
43. Leung DW, et al. Expression, purification, crystallization and preliminary X-ray studies of the Ebola VP35 interferon inhibitory domain. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 2009;65:163–5.

44. McKenna SA, et al. Purification and characterization of transcribed RNAs using gel filtration chromatography. *Nat Protoc* 2007;2:3270–7. [PubMed: 18079727]
45. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 1997;276:307–326.
46. Pflugrath JW. The finer things in X-ray diffraction data collection. *Acta Crystallogr D Biol Crystallogr* 1999;55:1718–25. [PubMed: 10531521]
47. Collaborative Computational Project, N. The CCP4 suite: programs for protein crystallography. *Acta Cryst* 1994;D50:760–763.
48. Vagin AA, Teplyakov A. MOLREP: an automated program for molecular replacement. *J Applied Crystallography* 1997;30:1022.
49. Read RJ. Pushing the boundaries of molecular replacement with maximum likelihood. *Acta Crystallogr D Biol Crystallogr* 2001;57:1373–82. [PubMed: 11567148]
50. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 1997;53:240–55. [PubMed: 15299926]
51. Lamzin VS, Wilson KS. Automated refinement of protein models. *Acta Crystallogr D Biol Crystallogr* 1993;49:129–47. [PubMed: 15299554]
52. Perrakis A, Sixma TK, Wilson KS, Lamzin VS. wARP: improvement and extension of crystallographic phases by weighted averaging of multiple-refined dummy atomic models. *Acta Crystallogr D Biol Crystallogr* 1997;53:448–55. [PubMed: 15299911]
53. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 2004;60:2126–32. [PubMed: 15572765]
54. Painter J, Merritt EA. Optimal description of a protein structure in terms of multiple groups undergoing TLS motion. *Acta Crystallogr D Biol Crystallogr* 2006;62:439–50. [PubMed: 16552146]
55. Davis IW, et al. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res* 2007;35:W375–83. [PubMed: 17452350]
56. Lee B, Richards FM. The interpretation of protein structures: estimation of static accessibility. *J Mol Biol* 1971;55:379–400. [PubMed: 5551392]
57. DeLano, WL. The PyMOL Molecular Graphics System. DeLano Scientific; San Carlos, CA, USA: 2002.
58. Delaglio F, et al. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 1995;6:277–293. [PubMed: 8520220]
59. Johnson BA. Using NMRView to visualize and analyze the NMR spectra of macromolecules. *Methods Mol Biol* 2004;278:313–52. [PubMed: 15318002]

Figure 1.

Overall structure of the VP35 IID-dsRNA complex. **(a)** Crystallographic asymmetric unit ($P2_12_12_1$) contains four VP35 IID molecules and one 8 base pair dsRNA. Two-fold non-crystallographic symmetry relates molecules A and C (green) and molecules B and D (cyan). The phosphate backbone of the dsRNA is represented by a magenta ribbon and the bases are shown in stick representation. **(b)** VP35 IID displays two different sets of interactions in the complex, where molecule A is mostly engaged in protein-protein interactions, while molecule B makes direct contacts with dsRNA. ITC binding isotherms and corresponding raw data for wildtype VP35 IID binding to **(c)** 8 base pair dsRNA and **(d)** 8 base single stranded RNA.

Figure 2. Conserved basic residues are important for protein-protein and protein-dsRNA interactions. (a-b), VP35 IID molecule A interacts with molecule B in a head to tail orientation, where residues Arg312 and Arg322 are involved in hydrogen bonding with the Asp271 and Glu262, respectively. (c-d) VP35 molecule B interacts with the dsRNA in a sequence independent manner with the sidechains of Arg312 and Arg322 forming hydrogen bonds with the phosphodiester backbone.

Figure 3.

The VP35 IID central basic patch residues are critical for dsRNA recognition. Electrostatic surface potential (scale of -10 to $+10 \text{ kT e}^{-1}$) of VP35 IID structures for (a) wildtype, (b) R312A, and (c) K339A. Locations of mutated residues are identified by green dashed circles. (d) Sites that are required for RNA binding and sites that enhance RNA binding are identified by red solid and dashed circles, respectively. The α -helical and β -sheet subdomains of VP35 IID are colored orange and yellow, respectively.

Figure 4.

Intersubdomain interface of VP35 IID forms an “end-cap” that recognizes blunt ends of duplex RNA. **(a)** Surface representation of VP35 IID (molecule B, cyan) and 8 base pair dsRNA (magenta) to highlight the surface complementarity between the 5' ends of dsRNA and VP35 IID β -sheet subdomain residues. Asterisks denote the complementary strand in the RNA duplex. **(b)** Residues Phe239 and Ile340 form van der Waals contacts with the 5' end of the C1 base (double arrow).

Figure 5.

Residues from the central basic patch and the "end-cap" of VP35 IID play key roles in the IFN-antagonist function. Sendai virus (SeV) infection-induced IFN- β promoter activity of VP35 IID wildtype (WT) and mutants are reported relative to IFN- β induction of an empty vector (EV) infected with SeV. **(a)** Residues from the first basic patch. **(b)** Residues from the "end-cap" (Phe235 is near Phe239, but is not part of the residues that form the "end-cap"). **(c-d)** Residues from the central basic patch. Error bars represent s.d. Plasmid concentration was 500 ng and the corresponding western blots are shown in Supplementary Fig. 9.

Figure 6.

VP35 competes with RIG-I for dsRNA binding and inhibits RIG-I dependent IFN- β promoter activation by dsRNA. 1H/15N HSQC of 15N-labeled VP35 IID (black) and in the presence of unlabeled (a) IVT-8 bp dsRNA (red), (b) IVT-8 bp dsRNA + RIG-I C-terminal domain (green), and (c) RIG-I C-terminal domain (blue). NMR signal for residue Arg312 is shown in the inset. (d) Comparison of VP35 IID residue number versus relative peak intensities of chemical shifts in a-c. (e) IVT-hairpin dsRNA activates IFN- β promoter in a RIG-I dependent manner. (f) VP35 can inhibit IFN- β promoter activated by RIG-I upon transfection of IVT-8 bp dsRNA and IVT-hairpin dsRNA. Error bars represent s.d.

Table 1

Data collection and refinement statistics

	VP35 IID/dsRNA complex	VP35 IID/dsRNA complex	VP35 IID R312A	VP35 IID K339A
Data collection				
Space group	<i>P2₁2₁2₁</i>	<i>P4₃2₁2</i>	<i>P6₁22</i>	<i>P2₁</i>
Cell dimensions				
<i>a</i> , <i>b</i> , <i>c</i> (Å)	78.95, 84.57, 89.32	81.03, 81.03, 90.25	81.55, 81.55, 343.49	53.38, 91.88, 102.60
α , β , γ (°)	90, 90, 90	90, 90, 90	90, 90, 120	90, 90, 99.6
Resolution (Å)	50.00-2.00 (2.03-2.00) *	50.00-2.40 (2.42-2.40) *	50.00-1.95 (1.98-1.95) *	50.00-2.40 (2.42-2.40) *
<i>R</i> _{sym} or <i>R</i> _{merge}	12.1 (75.8)	15.0 (87.6)	9.2 (n/a) ^a	4.4 (43.3)
<i>I</i> / σ <i>I</i>	15.9 (2.2)	21.3 (3.8)	27.0 (2.3)	29.0 (2.8)
Completeness (%)	100.0 (100.0)	100.0 (100.0)	99.6 (100.0)	99.8 (100.0)
Redundancy	4.8 (4.7)	14.0 (13.6)	11.4 (10.7)	3.8 (3.7)
Refinement				
Resolution (Å)	44.68-2.00	48.39-2.40	40.79-1.95	34.0-2.40
No. reflections	41,135	12,297	50,802	38,190
<i>R</i> _{work} / <i>R</i> _{free}	16.9/20.7	17.9/25.4	17.6/22.9	20.4/27.4
No. atoms				
Protein	3,862	1,937	3,985	5,685
RNA	336	168	n/a	n/a
Water	335	233	330	499
<i>B</i> -factors				
Protein	22.6 (chain A)	11.7 (chain A)	25.0 (chain A)	13.4 (chain A)
	20.3 (chain B)	7.1 (chain B)	23.0 (chain B)	21.5 (chain B)
	21.4 (chain D)		23.4 (chain C)	21.2 (chain C)
	21.3 (chain E)		24.6 (chain D)	23.8 (chain D)
				36.6 (chain E)
				32.8 (chain F)
RNA	10.8 (chain C)	5.2 (chain C)	n/a	n/a
	9.9 (chain F)			
Water	26.2	8.8	30.8	21.3
R.m.s. deviations				
Bond lengths (Å)	0.011	0.010	0.016	0.006
Bond angles (°)	1.23	1.22	1.44	0.91

Each dataset above was collected using one crystal.

* Values in parentheses are for highest-resolution shell.

^a If *R*_{merge} exceeds 1.0, Scalepack does not report its value because it is non-informative. Instead *I*/ σ (*I*) criterion is used to define resolution cut-off.

Table 2

Summary of dsRNA binding, suppression of IFN activation, and replication data

VP35 protein	dsRNA binding ¹	suppression of IFN activation ²	mini genome replication ³
WT	+++	+++	+
F235A	+++	+++	-
F239A	-	±	+
H240A	-	±	n.d. ⁴
R305A	+++	+++	+
K309A	++	++	+
R312A	-	+	+
K319A	++	++	+
R322A	-	±	+
K339A	-	±	+

¹ dsRNA binding measured by ITC experiments for VP35 IID (215-340) construct; +++, WT binding; ++, reduced relative to WT by a factor of 3-5; -, no observable binding

² suppression of IFN induced by Sendai virus (SeV) by full length VP35 (1-340) construct; +++, wildtype activity; ++, moderate inhibition; weak inhibition; ±, very weak inhibition.

³ minigenome replication data measure EBOV polymerase co-factor function of full length VP35 (1-340) construct; +, replication function detected; -, no replication function detected.

⁴ n.d., not determined. H240A was not expressed to detectable levels in this assay.